Gene Transfer May Be Preventive But Not Curative for a Lysosomal Transport Disorder

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Cystinosis belongs to a growing class of lysosomal storage disorders (LSDs) caused by defective transmembrane proteins. The causative *CTNS* gene encodes the lysosomal cystine transporter, cystinosin. Currently the aminothiol cysteamine is the only drug available for reducing cystine storage but this treatment has non-negligible side effects and administration constraints. In this study, for the first time, we report viral vector–mediated *CTNS* gene transfer and evaluate the feasibility of this strategy as a complementary treatment. Initially, we transduced human *CTNS*$^{-/-}$ fibroblast cell lines and primary murine *Ctns*$^{-/-}$ hepatocyte cultures *in vitro* and demonstrated that gene transfer can reduce cystine storage. Because of age-related increase in cystine levels, we transduced hepatocytes from young (≤3 months of age) and older (≥5 months of age) mice. Our *in vitro* data suggested that the efficiency of correction was age-dependent. We tested these observations *in vivo*: short-term (1 week) and long-term (4 weeks) *CTNS*-transduction significantly reduced hepatic cystine levels in young, but not older, *Ctns*$^{-/-}$ mice. Our data provide the proof-of-concept that gene transfer is feasible for correcting defective lysosomal transport, but suggest that, in the case of cystinosis, it could be preventive but not curative in some tissues.

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INTRODUCTION

Lysosomal storage disorders (LSDs) are a group of metabolic disorders characterized by a disruption of lysosomal function, which leads to the storage of diverse molecules.[1] In the majority of cases, the defect lies in one of the >50 lysosomal acid hydrolases that degrade most macromolecules. LSDs caused by defective hydrolases are considered excellent candidates for gene therapy.[2] First, they are monogenic, autosomal recessive disorders. Therefore, because the parents of an affected child are generally heterozygotes, this argues that low enzyme levels (as low as 10% of normal)[3] are sufficient to prevent a clinical phenotype. The second appealing aspect is the phenomenon of cross-correction whereby lysosomal enzymes can be secreted from one cell and taken up by neighboring cells.[4] Therefore, successful gene transfer to a relatively small subset of cells may be sufficient to reduce lysosomal storage throughout a tissue or organ.

The situation is more complex if an LSD is not caused by defective hydrolase activity. A number of LSDs are caused by defects in post-translational processing or trafficking of lysosomal hydrolases, or defects even in non-enzymatic proteins such as lysosomal transmembrane proteins.[1] Transmembrane proteins represent the newest and most rapidly expanding group of molecular defects giving rise to LSDs, and the functions of most of its members are still unclear. The two best-characterised proteins in this group are the lysosomal membrane transporters for cystine[5] and sialic acid,[6] which give rise to two LSDs: cystinosis and sialic acid storage disease, respectively.

The lysosomal cystine transporter is cystinosin, a predicted seven transmembrane domain protein encoded by the gene *CTNS*.[7] *CTNS* mutations lead to a spectrum of disease phenotypes, delineated into three main groups on the basis of age of onset and severity of the symptoms: infantile, juvenile, and ocular cystinosis.[8] Generally, cystine transport is abolished in infantile cystinosis whereas residual activity (9–20%) gives rise to the milder forms.[9] Clinically, like most LSDs, cystinosis is a multisystemic disorder.[8] The infantile form generally appears between 6 and 12 months of age with a proximal renal tubulopathy (Fanconi syndrome, characterized by fluid and electrolyte loss) and severe growth retardation. Corneal anomalies, notably photophobia caused by the formation of corneal cystine crystals at elevated free-cystine concentrations, appear from the age of 3 years and worsen with time. In the absence of treatment, end-stage renal disease can occur before 10 years of age. After successful renal transplantation, continuous widespread cystine accumulation leads to multiple complications (retinal, endocrinological, hepatic, gastrointestinal, muscular, and neurological). Juvenile cystinosis appears during adolescence with photophobia and renal glomerular impairment, but with a slower progression toward end-stage renal disease. The ocular form is characterized by corneal crystals with or without photophobia, and absence of renal dysfunction.

The sole treatment currently available to reduce lysosomal cystine levels is the aminothiol cysteamine.[10] Cysteamine administered in capsules early in the clinical course, in high doses, and

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every 6 hours delays progression to end-stage renal disease but it is ineffective against the Fanconi syndrome.[11,12] Although oral cysteamine can successfully treat certain systemic complications,[13,14] it does not reach the cornea. Therefore, cysteamine eye drops need to be administered 12 times a day to dissolve corneal crystals.[15] Data are unavailable on whether cysteamine retards or prevents neurological deterioration. Finally, cysteamine-treated patients have digestive intolerance (nausea, vomiting, and abdominal pain) and exude a nauseating odor.[16] Consequently, poor patient compliance renders the treatment significantly less effective.

The absence of an animal model for cystinosis had hindered the development of novel therapies. Therefore, in 2002, we generated mixed background 129Sv/C57BL/6 knockout (*Ctns*$^{-/-}$) mice[17] with elevated cystine levels, from birth, in the liver, kidney, lung, spleen, muscle, and brain. Subsequently, we generated congenic C57BL/6 *Ctns*$^{-/-}$ mice and showed that the temporospatial ocular phenotype mimics that of cystinosis patients.[18] In addition, we detected age-related learning and memory deficits that are reminiscent of the memory deficits reported in affected children.[19] Finally, in contrast to the mixed background mice, we recently found that the congenic *Ctns*$^{-/-}$ mice develop renal lesions, which are characterized by focal tubular atrophy predominately affecting the proximal tubules. These lesions worsen with age, leading to end-stage renal disease from 15 months of age (M. Chol, N. Nevo, A. Bailleux, V. Kalatzis, A. Onetti-Muda, M.-C. Gubler *et al.*; manuscript in preparation).

The existence of a disease model and the limitations of cysteamine treatment have prompted us to investigate novel avenues of therapy for cystinosis. Here, we report the first *in vitro* and *in vivo* viral vector–mediated gene transfer studies for cystinosis and the feasibility of reducing lysosomal cystine levels using this strategy. Our results show that *CTNS* gene transfer can reduce cystine storage caused by a defective lysosomal transporter, but suggest that this strategy may be preventive but not curative in some tissues. Given that *Ctns*$^{-/-}$ mice constitute the only animal model for defective lysosomal transport to date, our data from this model may be extrapolated to the disease group in general.

RESULTS

Lysosomal cystine reduction is feasible using *CTNS* gene transfer *in vitro*

We generated a canine adenovirus serotype 2 (CAV-2) vector expressing *CTNS* (CAVCTNS) and human adenovirus serotype 5 (Ad5) vectors expressing *CTNS* (AdCTNS) or *CTNS* fused to *GFP* (AdCTNSGFP). We used green fluorescent protein (GFP)-expressing CAV-2 (CAVGFP) and Ad5 (AdGFP) vectors as vehicle controls. We tested the *CTNS*-expressing viral vectors for production of cystinosin or cystinosin–GFP fusion protein by western blot analysis. A characteristic smear between ~50 and 60 kd corresponding to cystinosin in its various glycosylated forms was produced from CAVCTNS (**Figure 1a**) and AdCTNS (**Figure 1b**). A higher molecular weight smear (~75–85 kd) was produced from AdCTNSGFP (**Figure 1b**), which corresponded to the cystinosin–GFP fusion protein.

We then tested the feasibility of reducing lysosomal cystine levels by gene transfer to a *CTNS*$^{-/-}$ fibroblast cell line. In order

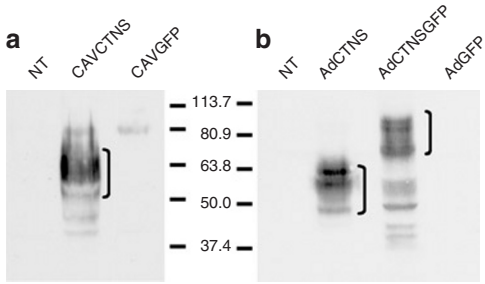

Figure 1 Verification of the expression cassette of the adenoviral vectors using western blot. (**a**) Cystinosin expression in nontransduced (NT) DKCre cells and in DKCre cells incubated with CAVCTNS or CAVGFP as a negative control. Because of its 7 N-glycosylation sites, cystinosin migrates as a smear between ~50 and 60 kd (bracket). (**b**) Cystinosin expression in NT 911 cells and in 911 cells transduced with AdCTNS (~50 and 60 kd; bracket), AdCTNSGFP (~75–85 kd; bracket), or AdGFP as a negative control. Ad, human adenovirus; CAV, canine adenovirus; GFP, green fluorescent protein.

to maximize Ad-mediated transfer efficiency in these cells, we pretreated them with the histone deacetylase inhibitor FR901228 (FR).[20–22] After FR-treatment, we observed an increase in transduction efficiency from 3 (not shown) to 50% (**Figure 2a**) as assayed by immunofluorescence using an anti-cystinosin antibody. Consistently, we significantly reduced cystine levels 48 hours after CAVCTNS transduction of FR-treated *CTNS*$^{-/-}$ fibroblasts, as compared to FR-treated, nontransduced *CTNS*$^{-/-}$ cells (**Figure 2b**). Cystine levels were not reduced to those of wild type (see **Table 1** for cystine content of control cells). We observed a small reduction that was not statistically significant after transduction with CAVGFP. We performed a time-course assay, and observed that cystine levels were not substantially lower at 72 or 96 hours after transduction as compared to the levels at the 48-hour time-point (**Figure 2c**). Therefore the 48-hour time-point was used for all subsequent experiments.

Taken together, these results suggest that *in vitro CTNS* gene transfer can reduce lysosomal cystine storage caused by defective cystine efflux.

The efficiency of cystine reduction is age-dependent

In vitro *analysis in primary hepatocyte cultures*. We next tested the feasibility of cystine reduction by *CTNS* gene transfer to primary cell cultures. Because the mouse liver highly expresses the coxsackievirus and adenovirus receptor,[23] we generated primary hepatocyte cultures from *Ctns*$^{-/-}$ mice of different ages. Regardless of the age of the mouse from which the hepatocyte cultures were generated, we obtained a transduction efficiency of >60% (**Figure 2d**). After CAVCTNS-transduction of primary hepatocytes from *Ctns*$^{-/-}$ mice aged ~3 months, we found a significant reduction in cystine levels as compared to nontransduced or CAVGFP-transduced cells (**Figure 2e**). Moreover, the cystine levels were comparable to those of wild-type cells (see **Table 1** for cystine content). However, CAVCTNS-transduction of primary hepatocytes from *Ctns*$^{-/-}$ mice aged ~6 months resulted in a significant but lower reduction in cystine levels (**Figure 2f**).

Our observations suggest that the efficiency of cystine clearance by *CTNS* gene transfer may be age-dependent in hepatocytes.

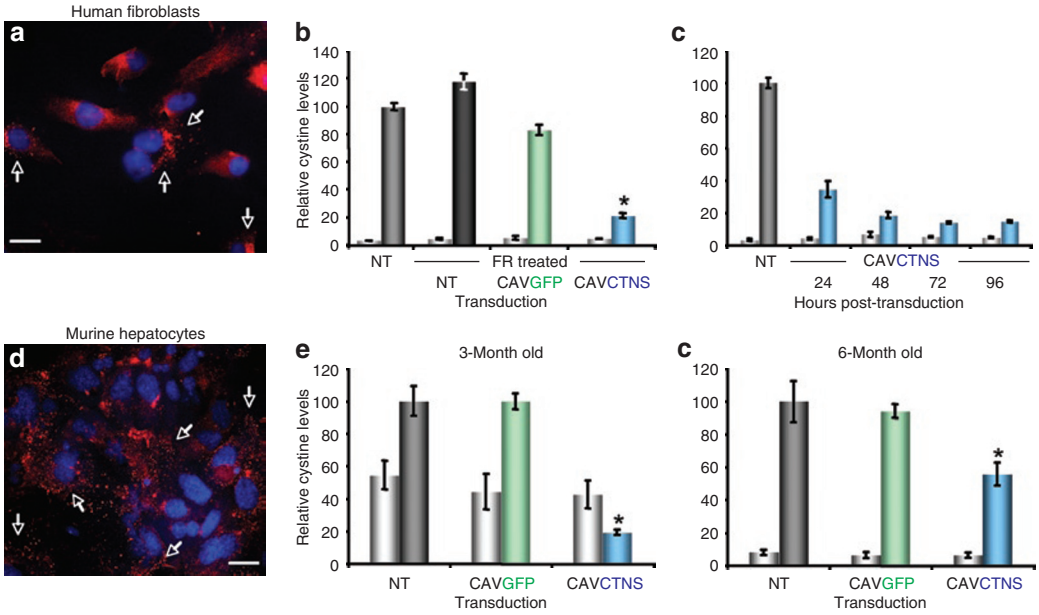

Figure 2 *In vitro* **gene transfer in (a–c) human fibroblast cell lines and (d–f) primary murine hepatocyte cultures.** (**a**) Immunofluorescence (IF) studies with an anti-cystinosin antibody shows lysosomal cystinosin expression (in red, arrows; nuclei in blue) in 50% of the fibroblasts treated with FR901228 (FR) and transduced with CAVCTNS. Bar = 20 µm. (**b**) Relative cystine levels in $CTNS^{+/+}$ (white bars) and $CTNS^{-/-}$ (colored bars) fibroblast cell lines expressed as percentages of nontransduced (NT) $CTNS^{-/-}$ levels (gray bar). Significant fivefold reduction in cystine levels in $CTNS^{-/-}$ fibroblasts after 24-hour treatment with FR and 48-hour transduction with CAVCTNS (blue bar). A nonsignificant 1.4-fold reduction is observed after transduction with CAVGFP (green bar). KW = 10.38, $P < 0.05$. *$P < 0.05$ versus FR-treated NT $CTNS^{-/-}$ fibroblasts (black bar). (**c**) Time course assay after CAVCTNS transduction of FR-treated $CTNS^{-/-}$ fibroblasts (blue bars). Cystine levels are expressed as percentages of NT $CTNS^{-/-}$ levels (in gray bars). In **b** and **c**, the data are presented as mean values ± SEM ($n = 3$). (**d**) IF studies with an anti-cystinosin antibody show lysosomal cystinosin expression (in red, arrows; nuclei in blue) in primary hepatocytes from 3-month-old mice transduced with CAVCTNS. Bar = 40 µm. (**e**) Relative cystine levels in $Ctns^{+/+}$ (white bars) and $Ctns^{-/-}$ (colored bars) primary hepatocyte cultures from ~3-month-old mice, expressed as percentages of NT $Ctns^{-/-}$ levels (gray bar). Significant fivefold reduction in cystine levels after a 48-hour transduction with CAVCTNS (blue bar). Cystine levels were not reduced after transduction with CAVGFP (green bar). KW = 11.81, $P < 0.05$. *$P < 0.05$ versus NT $Ctns^{-/-}$ hepatocytes. The data are presented as mean values ± SEM ($n = 5$ for NT and CAVGFP, $n = 7$ for CAVCTNS). (**f**) Cystine levels in $Ctns^{+/+}$ (white bars) and $Ctns^{-/-}$ (colored bars) primary hepatocyte cultures from ~6-month-old mice, expressed as percentages of NT $Ctns^{-/-}$ levels (gray bar). Significant twofold reduction in cystine levels after a 48-hour transduction with CAVCTNS (blue bar). Cystine levels were not reduced after transduction with CAVGFP (green bar). KW = 20.9, $P < 0.05$. *$P < 0.05$ versus NT $Ctns^{-/-}$ hepatocytes. The data are presented as mean values ± SEM ($n = 4$). CAV, canine adenovirus.

In vivo *analysis in the liver.* In order to further investigate the age-dependent phenomena observed *in vitro* in primary hepatocyte cultures, we targeted the livers of young (2- and 3-month-old) and older (6- and 9-month-old) $Ctns^{-/-}$ mice. Current data indicate that CAV-2 vectors inefficiently transduce the liver *in vivo* (E.J.K., unpublished results). Therefore, in order to obtain optimal liver transduction, we used Ad5 vectors for this study. We evaluated transduction efficiency by epifluorescence studies of GFP expression from AdGFP or AdCTNSGFP on liver sections. Regardless of age or vector, the number of transduced hepatocytes was, on average, 50% of the total (range of 20–75% depending on the mouse; **Figure 3a** and **c**). With respect to hepatic cystine levels (see **Table 1**), we detected statistically significant differences between the three experimental groups of young mice [2-month-old: KW = 10.43, $P < 0.05$ (data not shown) and 3-month-old: **Figure 3b**]. In contrast, we did not detect significant differences between the three experimental groups of older mice [5-month-old: KW = 5.14, $P > 0.05$ (data not shown) and 9-month-old: **Figure 3d**]. First, using AdGFP, we observed a reduction in cystine levels in the groups of young mice (**Figure 3b**) and those of older mice (**Figure 3d**) at 1 week after transduction, but this was not statistically significant. The effect of this control vector on cystine levels is addressed later in this report. Second, using AdCTNS, we detected a significant decrease in cystine levels in young $Ctns^{-/-}$ mice at 1 week after transduction as compared to levels in nontransduced mice (**Figure 3b**). A similar reduction was seen after transduction with AdCTNSGFP (data not shown). In contrast, transduction of older $Ctns^{-/-}$ mice with AdCTNS (or AdCTNSGFP, data not shown) did not significantly reduce cystine levels beyond that observed with AdGFP transduction (**Figure 3d**).

Taken together, our *in vivo* observations confirm our *in vitro* data that the efficiency of cystine clearance over a short transduction period is age-dependent in the liver.

Reduction efficiency remains age-dependent despite a longer post-transduction period

A possible explanation for the age-dependent efficiency of cystine clearance is that a longer duration of cystinosin expression is required to reduce the higher cystine levels in older mice (two- to sevenfold higher than those of younger mice; see **Table 1**). However, in many instances, the administration of E1- and E3-deleted Ad5 vectors results in T-cell mediated destruction of transduced cells from 7 days.[24] Indeed, after a 1-month transduction in the absence of an immunosuppressive protocol, cystine levels were not reduced in AdCTNS- or AdCTNSGFP-transduced young $Ctns^{-/-}$ mice as compared to the levels in AdGFP-transduced

Table 1 Cystine content of wild-type and cystinotic cells and liver tissues

	Normal			Cystinotic		
	(nmol half-cystine/mg protein)					
	Median	Range	*n*	Median	Range	*n*
Fibroblast cell lines	0.27	0.13–0.61	27[a]	6.8	5.0–8.6	6[b]
Hepatocyte cultures						
3 Months	0.36	0.17–0.65	11[c]	1.2	1.0–1.7	5[d]
6 Months	0.46	0.14–1.83	29[e]	9.0	4.7–11.9	12[f]
Liver tissue						
2 Months	0.03	0.02–0.24	12[g]	14.5	8.8–23.5	9[h]
3 Months				18.3	14.7–21.1	4[i]
5 Months				35.5	20.6–76.3	8[j]
9 Months				68.6	47.8–98.3	3[k]
AdCTNS-transduced liver[l]						
2 Months	—	—	—	2.7	1.8–3.7	4
3 Months	—	—	—	2.9	1.0–3.6	4
5 Months	—	—	—	12.0	8.6–19.1	4
9 Months	—	—	—	38.2	27.7–49.1	4
Clodrolip-treated liver[m]						
2 Months	—	—	—	3.6	2.3–4.9	3
5 Months	—	—	—	17.7	10.7–21.1	3

[a]Corresponds to the raw data from all experimental $CTNS^{+/+}$ groups in **Figure 2b** and **c**. [b]Corresponds to the raw data from the control nontransduced $CTNS^{-/-}$ groups in **Figure 2b** and **c**. [c]Corresponds to the raw data from all experimental $Ctns^{+/+}$ groups in **Figure 2e**. [d]Corresponds to the raw data from the control nontransduced $Ctns^{-/-}$ group represented in **Figure 2e**. [e]Corresponds to the raw data from all experimental $Ctns^{+/+}$ groups in **Figure 2f**. [f]Corresponds to the raw data from the control nontransduced $Ctns^{-/-}$ group represented in **Figure 2f**. [g]Corresponds to the $Ctns^{+/+}$ mice of all ages used in the short-term transduction experiments. [h,i]Corresponds to the 2- and 5-month-old nontransduced and nontreated $Ctns^{-/-}$ mice used in the short-term transduction and clodrolip experiments, respectively. [i,k]Corresponds to the 3- and 9 month-old nontransduced $Ctns^{-/-}$ mice used in the short-term transduction experiments. [l]Corresponds to the raw data from the AdCTNS-transduced $Ctns^{-/-}$ mice in the short-term experiments. The data for the 3- and 9-month-old mice are also represented graphically in **Figure 3b** and **d**, respectively. [m]Corresponds to the raw data from the $Ctns^{-/-}$mice 1 week after clodrolip treatment. The data for the 5-month-old mice are also represented graphically in **Figure 6c**.

mice (KW = 3.66, $P > 0.05$; data not shown). Therefore, in order to inhibit the T-cell response and extend the transgene expression period, a mild immunosuppression protocol using cyclosporin A (CsA) was carried out. A whole blood immunoassay demonstrated that CsA was continually administered over the 28-day period, although cyclosporinemia levels were lower at the time the animals were killed than at the beginning of the treatment (**Figure 4a**). T-cell infiltration was examined by immunohistochemistry (IHC) of liver sections, using an antibody against the T-cell marker CD3. At 1 week after transduction, a strong T-cell infiltration could be seen in mice that had not been treated with CsA (**Figure 4b**). A less intense infiltration could be seen at 1 month after transduction in nontreated mice (**Figure 4c**). In contrast, we did not detect a T-cell infiltration at 1 month after transduction in CsA-treated mice (**Figure 4d**), thereby suggesting that T-cell maturation was blocked.

We injected 3- and 5-month-old CsA-treated $Ctns^{-/-}$ mice with AdGFP and AdCTNS and killed them 1 month later. The number of transduced hepatocytes was, on average, 35% of the total number of hepatocytes (range of 25–60%) as determined by epifluorescence studies of AdGFP (data not shown). In parallel, we injected young mice with AdCTNSGFP. Surprisingly, we could not detect cystinosin–GFP fluorescence at 1 month after the injection (data not shown). We also carried out IHC screening of sections using an anti-GFP antibody. Although we found a strong GFP expression from AdGFP at 1 month after transduction (**Figure 5a**), we could not detect the cystinosin–GFP fusion protein at that time-point (**Figure 5b**). In contrast, we detected a strong cystinosin–GFP expression in mice at 1 week after transduction (**Figure 5c**). Finally, IHC studies with an anti-cystinosin antibody showed a persistent cystinosin expression in hepatocytes at 1 month after transduction with AdCTNS (**Figure 5d**).

Interestingly, and consistent with our short-term *in vivo* transduction data, we observed significant reductions in cystine levels in the group of young mice (**Figure 5e**) but not in the group of older animals (**Figure 5f**). First, using AdGFP, we observed a reduction in cystine levels in both age groups, but this was not statistically significant. Although this finding was consistent with our short-term observations, the reduction in cystine levels caused by AdGFP was greater at 1 week than at 1 month (**Figure 5e** and **f**). Second, long-term transduction of young $Ctns^{-/-}$ mice with AdCTNS resulted in a significant decrease in cystine levels as compared to levels in noninjected mice (**Figure 5e**). A similar reduction was seen after transduction with AdCTNSGFP (data not shown). As was the case for AdGFP, the decrease observed with the *CTNS*-expressing vectors was greater at 1 week after the injection than at 1 month.

In contrast, long-term transduction of older immunosuppressed $Ctns^{-/-}$ mice with AdCTNS (or AdCTNSGFP; data not shown) did not significantly reduce cystine levels beyond that observed with the use of AdGFP (**Figure 5f**). To assay cystinosin activity using another approach, we used transmission electron microscopy (TEM) to detect cystine crystals. We found that cystine crystals were present in all the mice regardless of age and the vector injected. The crystals were predominately located in Kupffer cells, as noted previously[17] (data not shown), but were also detected within hepatocytes (**Figure 5g** and **h**).

In conclusion, our long-term transduction data in the liver continue to show an age-dependent reduction in cystine clearance and suggest that this phenomenon may be attributable to a factor other than duration of cystinosin expression or presence of cystine crystals.

Kupffer cell destruction and regeneration after transduction affect cystine levels

Contrary to our expectations, cystine levels were more efficiently reduced in short-term as opposed to long-term *CTNS* gene transfer experiments. Moreover, the nonspecific effect of AdGFP on cystine reduction was more pronounced at 1 week than at 1 month after transduction. These observations suggest that cystine levels increase between these two time-points. Intravenous injection of Ad5 vectors leads to rapid destruction of Kupffer cells.[25] The destruction of Kupffer cells could account for the decrease in cystine levels observed with AdGFP in the short-term transduction experiments. In turn, we hypothesized

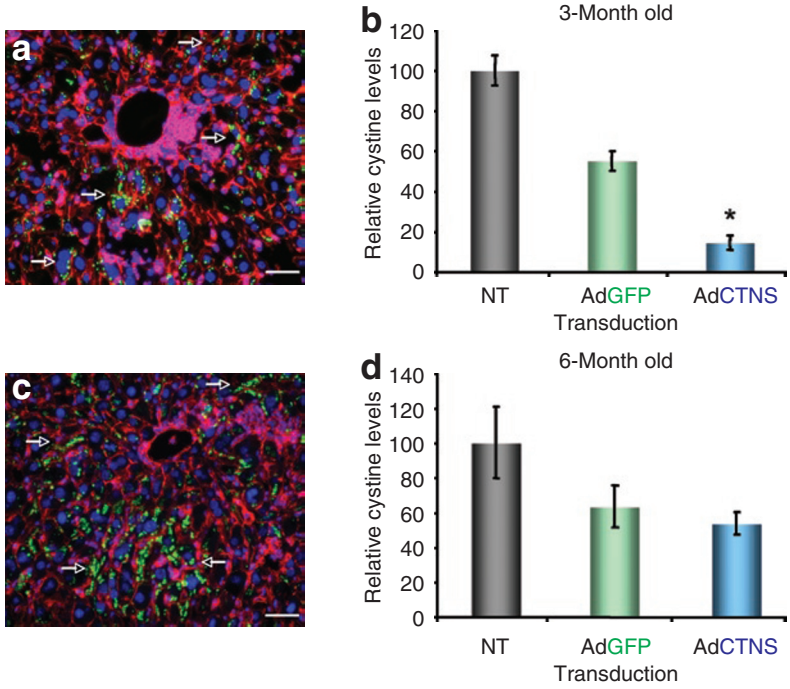

Figure 3 Short-term *in vivo* gene transfer to the liver in $Ctns^{-/-}$ mice. (**a**) Detection of green fluorescent protein (GFP) fluorescence from the cystinosin–GFP fusion protein (in green, arrows; nuclei in blue; plasma membrane in red) shows an *in vivo* transduction efficiency of 60% for AdCTNSGFP in a 2-month-old $Ctns^{-/-}$ mouse. (**b**) Significant sevenfold reduction in cystine levels [expressed as percentages of nontransduced (NT) levels] 1 week after transduction of 3-month-old $Ctns^{-/-}$ mice with AdCTNS. A nonsignificant 1.8-fold reduction was seen after transduction with AdGFP. KW = 12.73, $P < 0.05$. *$P < 0.05$ versus NT $Ctns^{-/-}$ mice. (**c**) GFP fluorescence from the fusion protein cystinosin–GFP (in green, arrows; nuclei in blue; plasma membrane in red) shows an *in vivo* transduction efficiency of 75% for AdCTNSGFP in a 9-month-old $Ctns^{-/-}$ mouse. Bars in **a** and (**c**) = 50 µm. (**d**) Nonsignificant 1.6- and 1.9-fold reductions in cystine levels 1 week after transduction of 9-month-old $Ctns^{-/-}$ mice with AdGFP and AdCTNS, respectively. KW = 4.91, $P > 0.05$. The data in **b** and **d** are presented as mean values ± SEM ($n = 4$). Ad, human adenovirus.

that a longer post-transduction period may result in the regeneration of Kupffer cells, leading to a rise in cystine levels.

In order to test this hypothesis, we artificially depleted Kupffer cells from 2- and 5-month-old $Ctns^{-/-}$ mice by treating them with liposomally encapsulated clodronate (clodrolip), a potent anti-macrophage agent.[26] IHC screening of liver sections using an antibody against the macrophage marker F4/80 demonstrated the absence (**Figure 6a**) and presence (**Figure 6b**) of Kupffer cells at 1 week and 1 month after clodrolip treatment, respectively. In parallel, an assay of hepatic cystine content showed that cystine levels were ~25% of those observed in nontreated mice 1 week after the administration of clodrolip, regardless of age (**Figure 6c**). In contrast, 1 month after treatment, cystine levels had risen to ~70% of the levels in nontreated animals (**Figure 6d**). In both cases, empty liposomes had no effect on cystine levels.

Taken together, these data suggest that Kupffer cell destruction consequent to Ad administration is responsible, in part, for the decrease in cystine levels 1 week after transduction, and explains the decrease observed with the use of AdGFP. In turn, Kupffer cell regeneration causes the increase in cystine levels seen at 1 month after transduction, perhaps partly accounting for the lower extent of decrease that we observed with AdCTNS at 1 month as compared to 1 week.

DISCUSSION

After the identification of *CTNS* in 1998, treatment by gene therapy became a potential option for cystinosis. In this study we have examined the feasibility of this approach *in vitro* and *in vivo* using Ad-mediated *CTNS* gene transfer.

Our approaches provide the proof-of-principle that it is feasible to use viral vector–mediated gene transfer for restoring a functional lysosomal membrane protein to a cell. It has been shown previously that stable transfection of human $CTNS^{-/-}$ fibroblasts with a *CTNS*-expressing plasmid reduces cystine levels by ≤70% without normalization.[27] Accordingly, although we were able to reduce cystine levels significantly *in vitro* in human $CTNS^{-/-}$ fibroblasts we were unable to achieve wild-type levels, likely because of the 50% transduction efficiency. Consistent with this hypothesis, we normalized cystine levels in readily transducible hepatocytes from young $Ctns^{-/-}$ mice *in vitro*. In contrast, *in vivo* transduction of the liver (with a hepatocyte transduction efficiency that was similar to that obtained *in vitro*) resulted in cystine levels that exceeded those of wild type by ~25-fold (as opposed to the 165-fold difference prior to transduction). This is likely due to the complexities of the *in vivo* system as compared to a simplified *in vitro* model. It is worth mentioning that in our initial *in vivo* experimental design we performed liver biopsies on each mouse 1 day before vector injection. In this way, we could directly compare the cystine content per mouse before and after vector transduction. However, the biopsy alone caused an ~50% reduction in hepatic cystine levels, as determined by a cystine assay 1 week after surgical intervention (data not shown). Although we did not examine this effect further, it may be attributable to a regeneration of the liver. In view of this finding, the

control hepatic cystine values for the *in vivo* experiments were obtained from age-matched $Ctns^{-/-}$ mice.

Our *in vitro* and *in vivo* data suggest that, at least in the cells and tissues assayed in our study, the efficiency of gene transfer in reducing lysosomal cystine levels is age-dependent. We initially suspected that a longer period of cystinosin expression was necessary to reduce the higher cystine levels in older mice. However, an extension of transgene expression from 1 week to 1 month was still not sufficient to significantly reduce cystine levels in older mice. One cannot, however, exclude the possibility that the lower proportion of transduced hepatocytes remaining at 1 month (35% at 1 month versus 50% at 1 week), coupled with the higher cystine levels in the surrounding nontransduced cells, may have masked the effect of *CTNS* gene transfer in older mice. We then examined the role of cystine crystals, which form at elevated cystine concentrations, and investigated whether the presence of crystals in older mice could interfere with cystine reduction. However, a TEM study showed that (i) the crystals were already present in young mice, and (ii) in both age groups crystals were detected only in a small number of hepatocytes in contrast to the predominant number of crystal-containing Kupffer cells. Although it seems unlikely, from the TEM data, that the age-dependent efficiency of cystine reduction was attributable exclusively to the presence of crystals, this assay was not quantitative. Therefore it cannot be ruled out that the hepatocytes of older $Ctns^{-/-}$ mice contained

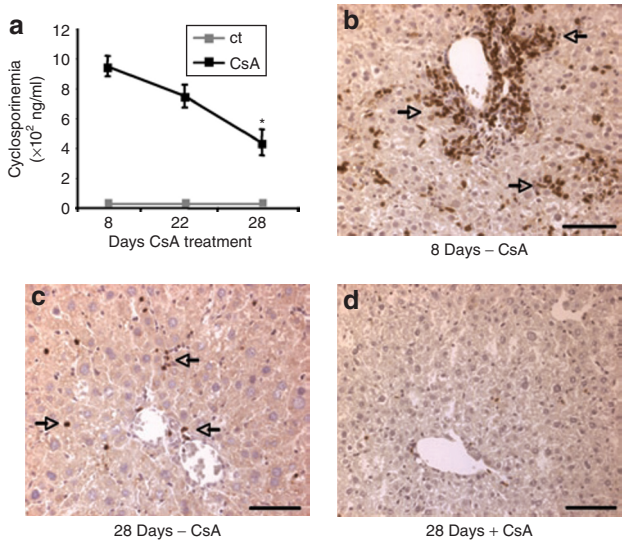

Figure 4 Evaluation of the immunosuppression protocol. (**a**) Cyclosporinemia values of cyclosporin A (CsA)-treated mice at 8, 22, and 28 days after treatment, showing that CsA was continually administered from the implanted osmotic pumps for the entire post-transduction period. Cyclosporinemia levels were significantly (twofold) lower at the time of killing the animal than at the start of the treatment. KW = 19.31, *$P < 0.05$ versus values at 8 days after the CsA treatment. The data are presented as mean values ± SEM of the cyclosporinemia values of mice from all experimental groups ($n = 23$). Control (ct) levels were <25 ng/ml. (**b**) Immunohistochemistry studies show evidence of a strong T-cell infiltration (in brown, arrows) at 1 week after transduction of a 2-month-old mouse not treated with CsA. (**c**) Weak T-cell infiltration (in brown, arrows) at 1 month after transduction of a 2-month-old mouse not treated with CsA. (**d**) Absence of T-cell infiltration at 1 month after transduction of a CsA-treated 2-month-old mouse (cyclosporinemia = 722 ng/ml). Bars in **b**, **c**, and **d** = 50 µm.

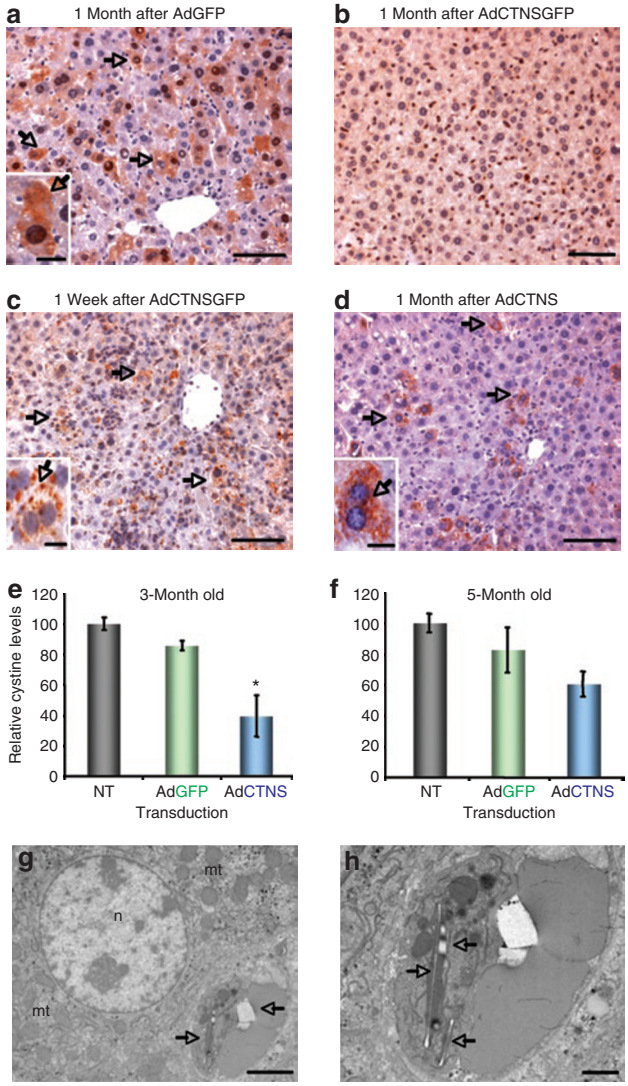

Figure 5 Long-term *in vivo* gene transfer to the liver in $Ctns^{-/-}$ mice. (**a**) Immunohistochemistry studies showing green fluorescent protein (GFP) expression 1 month after AdGFP-transduction of a 5-month-old cyclosporin A (CsA)-treated mouse (in red, arrows). Inset, higher magnification showing cytoplasmic GFP expression. (**b**) Absence of cystinosin–GFP expression at 1 month after AdCTNSGFP-transduction of a 3-month-old CsA-treated mouse. (**c**) Cystinosin–GFP expression at 1 week after AdCTNSGFP delivery to a young control mouse (in red, arrows). Inset, higher magnification showing cystinosin–GFP localized to large vesicles. (**d**) Cystinosin expression at 1 month after AdCTNS delivery to a 3-month-old CsA-treated mouse (in red, arrows). Inset, higher magnification showing a granular cystinosin expression. Bars in **a**–**d** = 50 µm. Bars in insets = 10 µm. (**e**) A significant (2.5-fold) reduction in cystine levels [expressed as percentages of nontransduced (NT) levels] at 1 month after AdCTNS delivery to 3-month-old CsA-treated $Ctns^{-/-}$ mice. A nonsignificant (1.2-fold) reduction was seen after injection with AdGFP. KW = 8.91, $P < 0.05$. *$P < 0.05$ versus NT $Ctns^{-/-}$ mice. (**f**) Nonsignificant (1.7- and 1.2-fold) reductions in cystine levels at 1 month after injection with AdCTNS and AdGFP, respectively, in 5-month-old CsA-treated $Ctns^{-/-}$ mice. KW = 5.11, $P > 0.05$. The data in **e** and **f** are presented as mean values ± SEM ($n = 4$). (**g**) Micrograph of a hepatocyte in a 5-month-old mouse at 1 month after AdCTNS injection, showing cystine crystals (arrows) contained in a multivesicular structure, which may represent an autophagolysosome (n—nucleus, mt—mitochondria). Bar = 3 µm. (**h**) Higher magnification of **g** showing a panoply of crystals contained within lysosomes. Bar = 1 µm. Ad, human adenovirus.

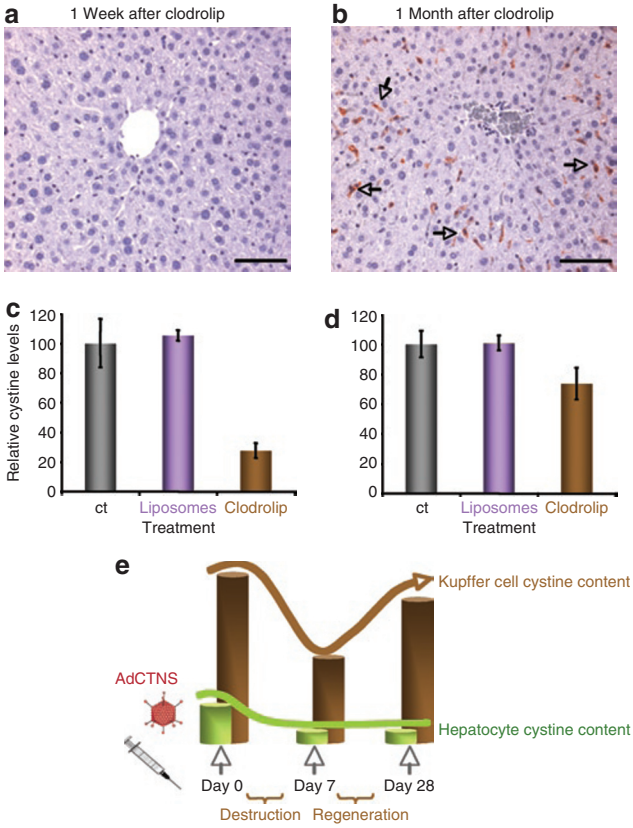

Figure 6 Depletion of Kupffer cells by use of clodrolip. Specific immunohistochemistry staining of Kupffer cells (arrows) using an antibody to the F4/80 macrophage marker on liver sections from mice killed at (**a**) 1 week or (**b**) 1 month after clodrolip treatment. The absence of staining in **a** confirms Kupffer cell depletion, whereas the staining in **b** demonstrates regeneration. Bars = 50 µm. (**c**) Cystine levels [shown as percentages of nontreated control levels (ct)] were 3.6-fold lower in 5-month-old mice at 1 week after clodrolip treatment, as compared to nontreated (ct) or liposome controls. (**d**) Cystine levels were 1.4-fold lower in 5-month-old mice at 1 month after clodrolip treatment than in controls. The data in **c** and **d** are presented as mean values ± SEM ($n = 3$). (**e**) Schematic representation of the evolution of hepatic cystine content after *CTNS* gene transfer over a 1-month period. Kupffer cells contain 75% hepatic cystine content at the time of injection, as determined by the 1-week clodrolip experiment. One week after the injection, the cystine content of Kupffer cells is reduced by ~50%, probably because of their destruction, as evidenced by the effect of AdGFP in the short-term transduction experiments. The cystine content of hepatocytes is reduced by ~70%, as evidenced by the difference between the effects of AdCTNS and AdGFP in the short-term experiments on young mice. One month after the injection, cystine levels in the Kupffer cells rise to ~80% of their original levels, as evidenced by the 1-month clodrolip experiment, and by the effect of AdGFP in the long-term transduction experiments. In contrast, hepatocyte cystine content remains similar, as indicated by the difference between the effect of AdCTNS and AdGFP in the long-term experiments on young mice. Ad, human adenovirus; GFP, green fluorescent protein

more crystals and that, as the free-cystine concentration began to decrease following *CTNS* transfer, the crystals began to dissolve, thereby causing free-cystine levels to rise again.

Our inability to achieve a reduction in cystine levels in older $Ctns^{-/-}$ mice suggests that the higher cystine content coupled with the rate of cystine being produced from metabolic breakdown or crystal dissolution exceeded the amount of cystine that cystinosin could remove in the given 1-month period. It could be envisioned that the higher the cystine levels, the longer the time necessary for cystinosin to remove a cystine build-up that is beyond steady state. Our results therefore suggest that gene transfer for cystinosis could be preventive but not curative and therefore, in order to ensure optimal efficiency, it should commence as soon as possible after diagnosis. This parallels the findings in the case of treatment with cysteamine, which is most effective if begun early in the clinical course of the disease.[11]

The liver was the organ of choice for the proof-of-principle study of gene transfer for cystinosis because of its accessibility *in vivo*. Interestingly, using the liver allowed us to serendipitously identify the population of cells responsible for hepatic cystine accumulation. The liver is made up of four cell populations: 65% are hepatocytes; 20% are endothelial cells; 10% are Kupffer cells, and the remaining 5% are stellate cells.[28] By artificially depleting the Kupffer cells, we depleted the liver of 75% of its cystine content. This is an important point to take into consideration *a posteriori*, given that Ad vector administration through the systemic circulation destroys Kupffer cells.[25] This accounts for the nonspecific 50% reduction in cystine levels observed with the use of the control AdGFP vector during the short-term transduction experiments (it is likely that the Ad vector was less efficient in destroying the Kupffer cells as compared to clodrolip). Although the high percentage of hepatic cystine contained in the minority Kupffer cell population was a surprising finding, the relatively higher cystine content of Kupffer cells as compared to other liver cell populations is consistent with the observations in patients with cystinosis. Generally, the cystinotic liver is histologically normal with the exception of enlarged Kupffer cells caused by crystal formation.[29] Moroever, the hypertrophic Kupffer cells may be the cause of the age-related portal hypertension reported in patients.[30,31]

Following depletion, Kupffer cell repopulation of the liver is complete after a further ~2 weeks.[26] At 1 month after clodrolip treatment, we showed that cystine levels in the treated animals rose from 25 to 70% of the levels in the controls. Consistently, the regeneration of Kupffer cells accounts, at least partly, for the higher nonspecific reduction in cystine levels (80% of control) observed at 1 month after the injection with AdGFP as compared to the reduction found at 1 week. However, even if we take into account the nonspecific reduction in cystine levels attributable to Kupffer cell destruction, and the subsequent increase caused by Kupffer cell regeneration, the cystine levels are still reduced specifically with the use of *CTNS*-expressing vectors, at least in young mice. First, in the short-term transduction experiments, AdCTNS-transduced livers show cystine levels that are 15% of those in the controls (as compared to 55% in AdGFP-transduced livers). Second, in the long-term AdCTNS-transduction experiments, cystine levels in the liver were 40% of those in the controls (as compared to 85% after AdGFP transduction). Taken together, these observations indicate that specific transduction is achieved by *CTNS* transfer and cystinosin expression, leading to a significant reduction in the cystine content of hepatocytes (**Figure 6e**, schematic representation).

Finally, our results relating to the duration of transgene expression are interesting to note. A 1-month transduction of immunocompetent mice with AdGFP or AdCTNS resulted in a persistent GFP or cystinosin expression, respectively. This was unexpected, given the well-documented T-cell mediated clearance of Ad-transduced

cells beginning from 1 week after transduction.[24,32,33] However, the response in mice to intravenous Ad vector administration is complex, and depends on the interplay between numerous parameters such as the mouse strain, the strength of the promoter, and the half-life and immunogenicity of the transferred protein, to mention only a few. In contrast, we could not detect expression of cystinosin fused to GFP. These observations were identical to those in immunosuppressed mice 1 month after transduction. Taken together, these results may indicate either that GFP-tagged cystinosin is degraded more rapidly or that it is more immunogenic.

We have consistently noted *in vitro* that cystinosin–GFP is found in large intracellular vesicles[5,9,34,35] whereas cystinosin alone localizes to small discrete vesicles (see the data provided); in both cases, the labeled vesicles were also positive for lysosomal-associated membrane protein 2, thereby confirming that they are lysosomes. It is therefore tempting to speculate that GFP-tagged cystinosin may provoke the fusion of lysosomes, or perhaps the creation of autophagolysosomes, which are then cleared by the cell. Consistently, we detected spurious bands of a smaller molecular weight in the western blot analysis of cystinosin–GFP expression from AdCTNSGFP. These may be indicative of the instability of this fusion protein. We included the AdCTNSGFP vector in this study as an internal marker for transgene expression. However, the lack of detectable cystinosin–GFP expression at 1 month after transduction suggests that future *CTNS* vector constructs should make use of an internal ribosome entry site sequence for GFP marker expression so as to avoid premature clearance of cystinosin.

Taken together, we have provided the proof-of-principle that viral vector–mediated gene transfer can correct a defect in lysosomal transport to reduce abnormal lysosomal storage. Numerous LSDs (such as Gaucher, Fabry, Pompe, Tay Sachs, and Mucopolysaccharidosis types I, II, II, VI, and VII) have been the focus of gene therapy studies with, in many cases, encouragingly positive results.[36,37] Although the target organ, vector, or model may have differed among these studies,[38] a common aspect of these diseases is that they are caused by defective lysosomal hydrolases and are therefore amenable to cross-correction. However, it is becoming increasingly evident that defective lysosomal hydrolase activity is not the sole, although it is the most frequent, cause of LSDs. Defects in soluble non-enzymatic lysosomal proteins have been reported to underlie four LSDs, and defects in transmembrane (non-enzymatic) proteins have been linked to another eight.[1] To our knowledge, the feasibility of gene transfer has not been previously addressed in either of these classes. Therefore our reported data of gene transfer using the cystinosis model could be extrapolated to other lysosomal transport disorders for which an animal model does not yet exist, such as the sialic acid storage disease group.

In terms of perspectives for cystinosis gene therapy, cystinosis is a multisystemic disease and unfortunately there is no single vector that transduces all tissues with equal efficiency. Nonetheless, in multisystemic diseases, there are organs that are affected more severely or earlier than others. In cystinosis, this organ is the kidney. However, given the complex architecture of this organ, kidney gene transfer is still in its infancy as compared to gene transfer to other organs. A particularly interesting and accessible tissue to target for cystinosis is the cornea, as it is affected early in the disease and the resulting lesions represent a major handicap and they are not ameliorated by oral cysteamine treatment.[39] Moreover, as the corneal anomalies in *Ctns*$^{-/-}$ mice mimic those in cystinosis patients,[18] this preclinical model is appropriate for evaluating corneal phenotypic correction. Although the E1–E3-deleted Ad vectors used in our study were suitable to test proof-of-concept, subsequent gene transfer studies should use more stable vectors such as helper-dependent Ad vectors, which are capable of extended transgene expression because of their lower immunogenicity.[40,41] By adopting this approach, we can more accurately evaluate the feasibility of gene transfer as a complementary treatment to oral cysteamine. In the long term, the emergence of new clinically relevant vectors with varied tropisms may make it possible to envisage gene transfer as an alternative treatment for cystinosis.

MATERIALS AND METHODS

Generation of CAVCTNS. The CTNS cDNA sequence was amplified from a λgt10 phage clone using arm primers. The PCR product was digested with *Xho*I (situated 250 bp upstream of the ATG start codon) and *Apa*I (situated 88 bp downstream of the TAG stop codon) and subcloned into the *Xho*I/*Apa*I-digested transfer plasmid pTCAV12a[42] to generate pTCAVCTNS. Subsequently, *Not*I-linearized pTCAVCTNS and *Swa*I-linearized pTG5412 containing the CAV-2 genome[42] were cotransformed into the recBC sbsBC strain of *Escherichia coli*, BJ5183 (ref. 43), at a 1:5 molar ratio. The resultant homologous recombination generated a 33-kb plasmid containing the E1-deleted (ΔE1) CAV-2 genome and the *CTNS* expression cassette (pCAVCTNS). Finally, *Not*I-digested pCAVCTNS was transfected into E1-transcomplementing DKCre[44] cells, using the conditions described earlier,[42] to produce CAVCTNS. CAVCTNS was purified using CsCl gradients (isopycnic gradient density 1.32 g/ml), and the titer was determined by optical density reading at 260 nm to be 1.4×10^{12} physical particles (pp)/ml. CAVGFP was generated previously[42] at a titer of 2.7×10^{12} pp/ml.

Generation of AdCTNS and AdCTNSGFP. The *CTNS* expression cassette was excised from the plasmid pcDNA-CTNS[34] by digestion with *BamH*I and *Xba*I, and subcloned into the *BamH*I/*Xba*I-digested transfer plasmid pTG6600 (Transgene, Strasbourg, France) to generate pTGCTNS. The expression cassette containing *CTNS* fused to *EGFP* at the 3′ end was excised from the plasmid pCTNS-EGFP-N1 (ref. 34) by digestion with *Xho*I and *Not*I and subcloned into the *Xho*I/*Not*I-digested pTG6600 to generate pTGCTNSGFP. Subsequently, *Fsp*I-linearized pTGCTNS or pTGCTNSGFP and *Swa*I-linearized pKP1.3 containing the E3-deleted (ΔE3) Ad5 genome, were cotransformed into BJ5183 at a 1:10 molar ratio to generate a 34-kb plasmid containing the ΔE1ΔE3 Ad5 genome and the CTNS (pKPCTNS) or CTNSGFP (pKPCTNSGFP) expression cassette, respectively. E1-transcomplementing 911 cells[45] (10^6 cells per well in 6-well plates) were then transfected with 4 μg *Pac*I-digested pKPCTNS or pKPCTNSGFP using 8 μl Lipofectamine 2000 (Invitrogen Life Sciences, Cergy Pontoise, France). The amplification steps using 911 cells, subsequent purification using CsCl gradients (isopycnic gradient density 1.34 g/ml), and titration of vector stocks were performed as for CAVCTNS.[42] The titers of both AdCTNS and AdCTNSGFP were 6.5×10^{12} pp/ml. AdGFP was generated previously[42] at 3.4×10^{12} pp/ml.

Western blot assays. For assaying cystinosin expression from the CAV-2 or Ad5 vectors, DKCre or 911 cells, respectively, were incubated with 100 pp/cell for 48 hours. The cells were washed and scraped in cold phosphate-buffered saline containing a protease inhibitor cocktail (Complete; Roche Diagnostics, Meylan, France). Following centrifugation, the cell pellet was resuspended in Laemmli's loading buffer containing Benzonase (Sigma-Aldrich, St. Quentin Fallavier, France) and loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel. The separated proteins were electrotransferred to nitrocellulose filters (Amersham GE Healthcare, Orsay, France). The filters were blocked in 20 mmol/l Tween/phosphate-buffered saline containing

5% dried milk overnight at 4°C and then incubated with 1:500 rabbit anti-human cystinosin antibody[35] for 1 hour at room temperature (RT). The washed membranes were then incubated with 1:10,000 anti-rabbit IgG–HRP antibody (Sigma-Aldrich) for 45 minutes at RT and detected using the ECL western blotting substrate (Amersham GE Healthcare).

Animals. Male *Ctns*$^{+/-}$ mice from the 129Sv/C57BL/6 mixed line[17] were backcrossed with wild-type C57BL/6 females for 10 generations at the Centre de Distribution, Typage et Archivage Animal (Orleans, France). *Ctns*$^{+/+}$ and *Ctns*$^{-/-}$ mice were then intrabred to generate pure litters at the Institut Génétique Moléculaire de Montpellier. All the mice were maintained in a controlled environment with a 12 hour/12 hour light/dark cycle, housed in groups of a maximum of 10, and allowed food and water *ad libitum*. The procedures were in accordance with the European Community Council Directive of 24 November 1986 (86-609).

Culture and transduction of CTNS$^{+/+}$ and CTNS$^{-/-}$ fibroblast cell lines. Skin fibroblasts were isolated from a normal individual (*CTNS*$^{+/+}$) and from an infantile cystinosis patient carrying a homozygote 57-kb deletion that removes most of *CTNS* (*CTNS*$^{-/-}$). Immortalization by the SV40 T-antigen[46] was performed at the Centre de Thérapie Génique (Nantes, France). Fibroblast cell lines were cultured (3×10^5 cells per well in a 6-well plate) in Dulbecco's modified Eagle medium containing GlutaMAX (Gibco-Invitrogen) supplemented with 10% fetal calf serum (Perbio Science, Bezons, France) at 37°C with 5% CO_2. Before being transduced, the fibroblasts were treated with 1.5 ng/ml FR (kindly provided by the Fujisawa, Tokyo, Japan) for 24 hours.[20–22] Treated cell lines were then washed and incubated with 1,000 pp/cell for 48 hours. All the experiments were performed in triplicate.

Isolation, culture, and transduction of primary Ctns$^{+/+}$ and Ctns$^{-/-}$ hepatocytes. Mice 3–4 months of age (two *Ctns*$^{+/+}$ and two *Ctns*$^{-/-}$) and 5–8 months of age (three *Ctns*$^{+/+}$ and four *Ctns*$^{-/-}$) were anesthetized by intraperitoneal injection of 10 mg/kg xylazine (Bayer Pharma, Puteaux, France) and 100 mg/kg ketamine (Merial, Lyon, France). Hepatocytes were isolated by sequential portal vein perfusion of (i) 6 UI/ml heparin/0.66 mmol/l EGTA, (ii) 10 mmol/l HEPES, 150 mmol/l NaCl, and 30 mmol/l KCl at pH 7.8, and (iii) 0.13 mg/ml collagenase A/10 mmol/l $CaCl_2$ (ref. 47). After repeated washing and purification on a Percoll density gradient (Amersham GE Healthcare), freshly isolated hepatocytes were cultured (3×10^5 cells per well in a 6-well plate) in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 0.1 µmol/l dexamethasone, 5 µg/ml insulin, and 0.5% dimethyl sulfoxide at 37°C with 5% CO_2. Twenty-four hours after plating, hepatocyte cultures were directly transduced with 1,000 pp/cell for 48 hours. All CAVCTNS transductions were performed at least in triplicate.

In vivo *transduction of Ctns$^{-/-}$ mice.* For the short-term transduction experiments, *Ctns*$^{-/-}$ mice 2, 3, 6, and 9 months of age were injected in the tail vein with 150 µl phosphate-buffered saline containing 5×10^{10} pp of AdCTNS, AdCTNSGFP, or AdGFP. Four mice were used per experimental condition. The mice were killed 7 days after injection. For the long-term transduction protocol, ALZET osmotic pumps (Charles River Laboratories, Lyon, France) filled with CsA (Sandimmun IV, kindly provided by Frederic Bernard, Hôpital Arnaud de Villeneuve, Montpellier, France) were implanted subcutaneously in anesthetized 3- and 5-month-old *Ctns*$^{-/-}$ mice, and the equivalent of 15 mg/kg/day of CsA was delivered through the pumps for a 28-day period. One day after the implantation, the mice were injected with 150 µl phosphate-buffered saline containing 5×10^{10} pp AdCTNS, AdCTNSGFP, or AdGFP in the tail vein, and killed 28 days after the injection. Four mice were used per experimental condition.

Kupffer cell depletion by liposomal clodronate. Liposomally encapsulated clodronate (clodrolip) was administered by intraperitoneal injection at a concentration of 2 mg/20 g in two groups of *Ctns*$^{-/-}$ mice of ages 2 and 5 months. Four days after the injection, a second dose of 1 mg/20 g of clodrolip was administered to ensure depletion of >90% of Kupffer cells. As a negative control, other mice were injected in parallel with empty liposomes. The mice were killed either 7 or 28 days after the first injection. Three mice were used per experimental condition.

Immunofluorescence studies. For the *in vitro* studies, fibroblasts and hepatocytes were cultured on glass coverslips. After transduction, coverslips were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.05% saponin/3% bovine serum albumin for 10 minutes. In order to detect cystinosin, coverslips were incubated with 1:500 anti-cystinosin antibody for 1 hour at RT, washed, and then incubated with 1:50 anti-rabbit IgG-TRITC antibody (Sigma-Aldrich) for 45 minutes. Nuclei were stained with 0.2 µg/ml bisBenzimide Hoechst (Sigma-Aldrich) for 5 minutes before being mounted (DakoCytomation fluorescent mounting media; DakoCytomation, Trappes, France). In order to detect GFP, nuclei were stained and the coverslips were directly mounted. For the *in vivo* studies, part of the liver was fixed in 4% paraformaldehyde for 24 hours, placed in 20% sucrose for 24 hours, and embedded in OCT matrix (CellPath, Powys, UK). For detecting GFP, permeabilized 5- to 10-µm-thick sections were incubated with 1 ng/ml phalloidin-TRITC (Sigma-Aldrich) and Hoechst for 15 minutes at RT before mounting. Transduction efficiency was determined by counting the number of GFP-expressing cells in relation to total cells, using the MetaMorph imaging program (Molecular Devices, Wokingham, UK).

IHC analysis. Paraformaldehyde-fixed liver samples were dehydrated in graded ethanol and embedded in paraffin (Histowax; Microm Microtech, Francheville, France). Four micrometer-thick sections were deparaffinized in xylene, rehydrated, incubated for 30 minutes at 100°C in citrate buffer, pH 6.0, and then for 10 minutes in 1% H_2O_2. Following three washes in 0.1% Tris-buffered saline–Tween, the sections were blocked with 20% horse serum for 30 minutes followed by avidin–biotin for 2 × 15 minutes (Vector Laboratories, Burlingame, CA). The blocked sections were incubated with 1:300 rat anti-mouse F4/80 antibody (Clone BM8; Caltag-Invitrogen) to detect Kupffer cells, 1:500 goat anti-mouse CD3 antibody (Clone M-20; Santa Cruz Biotechnology, Santa Cruz, CA) to detect T cells, 1:500 rabbit anti-GFP antibody (Abcam, Cambridge, UK) or 1:300 anti-cystinosin antibody for 1 hour at RT. After being washed, the sections were incubated with 1:300 anti-rat, 1:500 anti-goat, or 1:500 anti-rabbit IgG biotinylated antibodies (Vector Laboratories) for 1 hour at RT. The sections were stained using 1:100 ExtrAvidin Peroxidase (Sigma-Aldrich) with either 3-amino-9-ethylcarbazole (Sigma-Aldrich) or diaminobenzidine (exclusively for anti-CD3; Vector Laboratories) substrate and counterstained with hematoxylin. 3-Amino-9-ethylcarbazole sections were mounted directly in VectaMount aqueous mounting medium (Vector Laboratories). Diaminobenzidine sections were dehydrated and mounted in VectaMount permanent mounting medium.

TEM studies. To detect cystine crystals, part of the liver of each animal was fixed in 3.3% glutaraldehyde, post-fixed in 2% osmium tetraoxide, and embedded in epoxy resin (Electron Microscopy Sciences, Hatfield, PA). Eighty-five-nanometer-thick sections were stained with uranyl acetate and lead citrate, and visualized (Hitachi H7100).

Assay of cystine levels. For the *in vitro* studies, fibroblasts and hepatocytes were trypsinized and centrifuged, and the washed pellet was resuspended in 0.65 mg/ml N-ethylmaleimide (Sigma-Aldrich). For the *in vivo* studies, liver pieces were collected in N-ethylmaleimide and homogenized. After sonication of the cell or liver homogenates, proteins were precipitated and assayed as described previously.[19] The protein-depleted supernatant was assayed for cystine content by radio-competition[48] with ^{14}C-cystine (Perkin-Elmer Life Sciences, Villebon sur Yvette, France) for the cystine binding protein (Riverside Scientific Enterprises, Bainbridge Island, WA), as described earlier,[19] with one exception: the liver supernatants were diluted (1:100 to

1:500 for the 2- to 6-month-old mice, and 1:1,000 for the 9-month-old mice) before the assay. Assays were repeated three or more times per experiment. Data from one representative experiment are shown.

Cyclosporin whole blood immunoassays. A 200 µl sample of EDTA anticoagulated blood was taken from CsA-treated *Ctns*$^{-/-}$ mice (nontransduced and transduced; n = 23) and from nontreated, nontransduced control mice (n = 3) by retro-orbital puncture at 8 and 22 days after the implantation and at the time of killing (28 days). Blood CsA levels were analyzed using the EMIT 2000 cyclosporin-specific assay (Dade Behring, Paris, France); these analyses were performed by the Department of Biological Immunology, Hôpital Necker-Enfants Malades (Paris, France).

Statistical analysis. Given the small sample sizes, data were analyzed using a nonparametric Kruskal–Wallis ANOVA, and post-hoc comparisons were made using a Siegel–Castellan 2 × 2 comparison.[49]

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